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The development of a sensitive and specific enzyme immunoassay for FK480, a novel cholecystokinin type-A receptor antagonist, in human plasma

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Abstract

A sensitive and specific enzyme immunoassay for FK480, a novel cholecystokinin type-A (CCK-A) receptor antagonist, was developed to study the pharmacokinetics of the drug at low-dose administration using a specific monoclonal antibody. The high performance liquid chromatography (HPLC) method had been used for studying toxicokinetics, but its determination limit (2.5 ng ml^{-1}) was too high for use in clinical studies. Subsequently we developed an enzyme immunoassay (EIA) using rabbit anti-FK480 serum (polyclonal antibody). It had higher sensitivity (0.1 ng ml^{-1}) when 0.5 ml of plasma was used but its specificity was low because of the cross-reactivity of the metabolites of FK480. Therefore we produced several monoclonal antibodies for FK480 by cell fusion, and selected the antibody which was least cross-reactive for the isolated metabolites of FK480. Finally we developed a sensitive and specific EIA using this monoclonal antibody. The lower limit of quantification of this method was 0.2 ng ml^{-1} when 0.2 ml of human plasma was used. The coefficient of variation over the calibration range (0.2–10 ng ml^{-1}) was less than 15%. We used this method for clinical studies, and it showed a good correlation to the HPLC method when plasma concentration was 2.5 ng ml^{-1} or more. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: FK480; Cholecystokinin antagonist; Enzyme immunoassay; Metabolite; Cross-reactivity

1. Introduction

FK480 is a newly synthesized non-peptide CCK-A receptor antagonist which is orally effective and has higher activities than loxiglumide or other CCK antagonists [1,2]. The concentrations of loxiglumide in human plasma were measured by a high performance liquid chromatography (HPLC) method using C18 column with UV detection [3]. As the clinical dose of FK480 was considerably lower (i.e. 1 mg $body^{-1}$ or less) than that of loxiglumide, a sensitive assay method of FK480 in human plasma was needed to study its pharmacokinetics. Enzyme immunoassay (EIA) has been a useful and sensitive method of drug

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assay in biological fluids. One of the important issues for EIA is specificity of the used antibody to antigen, i.e. drug. Especially, care must be taken of the cross-reactivity of metabolites when the method is used for a pharmacokinetic study. In the case of immunoassay for hapten (low molecular weight chemicals), the following methods have been studied to improve specificity: (i) changing the binding site to carrier protein and enzyme [4,5]; (ii) involving the extraction procedure, for example, organic solvent extraction [6,7], solid phase extraction [8] or HPLC-Immunoassay [9,10]; and (iii) using specific monoclonal antibody [11–15]. The latter will be the most effective if a sensitive and specific monoclonal antibody can be selected.

This report describes: (i) the validation of the HPLC and EIA using polyclonal antibody (PAb); (ii) the selection of a less cross-reactive monoclonal antibody to isolated metabolites; and (iii) the successful development of a sensitive and specific EIA of FK480 incorporated with both monoclonal antibody (MAb) and solvent extraction. It also describes the application of this assay in a clinical study.

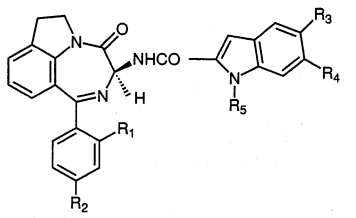
2. Materials and methods

2.1. Reagents and buffer

FK480, internal standard (FR122947) and its metabolites M1 (FR189433) and M2 (FR189435) were synthesized by Fujisawa Pharmaceutical Company (Fig. 1). Polyethylene glycol (PEG) 4000 was purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA, fraction V), tween-20, pristane, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and Freund's adjuvant were purchased from Nacalai Tesque (Kyoto, Japan). Skim milk was obtained from Difco (Detroit, MI). Horseradish peroxidase (POD) and o-phenylene diamine hydrochloride tablets were obtained from Sigma (St. Louis, MO). All other chemicals used were guaranteed reagent grade. Anti-mouse IgG (Hand L specific) was purchased from Atlantic Antibody (USA) and POD-labeled anti-mouse IgG purchased from Jackson ImmunoResearch (West Grove, PA). Phosphate buffer saline (pH 7.4, 0.15 M NaCl, 10 mM phosphate buffer; PBS) was used as coating buffer. The blocking buffer was 1% BSA or 5% skim milk in PBS. The washing solution was PBS with 0.05% tween-20. Cell culture medium, RPMI-1640 was from Nikken Biomedical Laboratories (Kyoto, Japan).

2.2. HPLC method

Plasma (0.5 or 1 ml for dog or human, respectively) was extracted with 4 ml of 2% isoamyl alcohol in n-hexane under basic condition after adding internal standard solution. The mixture was shaken for 10 min, followed by centrifugation for 5 min at 3000 rpm (ca 2000 g). For the dog plasma measurement the upper organic layer (3 ml) was evaporated to dryness under a nitrogen stream. For the human plasma measurement the following solid phase extraction was needed to avoid interference from the control plasma components. The upper organic layer (3 ml) was applied directly to Bond Elut silica cartridge (500 mg/3 ml, Varian, CA) which had been equilibrated with 3 ml of n-hexane. The cartridge was washed with 9 ml of n-hexane-diethyl ether (50:50, v/v). The adsorbed FK480 was eluted



Substance	R_1	R_2	R_3	R_4	R_5
FK480	F	H	H	H	H
Internal standard (FR122947)	H	CH_3	H	H	H
M1 (FR189433)	F	H	OH	H	H
M2 (FR189435)	F	H	H	OH	H
Immunogen	F	H	H	H	-$(CH_2)_3$CO-BSA
Enzyme conjugate	F	H	H	H	-$(CH_2)_3$CO-POD

Fig. 1. Structures of FK480 and related compounds.

Table 1
Intra-day precision and accuracy measurements of HPLC method for FK480

Plasma	Concentration spiked (ng ml^{-1})	Concentration found (ng ml^{-1})	n	Coefficient of variation (%)	Bias (%)
Dog	3	2.8 ± 0.2[a]	5	7.3	−6.7
	50	52.5 ± 1.3	5	2.4	5.0
	1000	1002.2 ± 9.3	5	0.9	0.2
Human	2	1.80 ± 0.15	5	8.7	−10.0
	20	20.03 ± 0.80	5	4.1	0.1

[a] Mean ± S.D.

from the cartridge with 6 ml of n-hexane-diethyl ether (20:80, v/v) and evaporated to dryness under a nitrogen stream. Both resulting residues from dog and human plasma were reconstituted in 200 μl of mobile phase and analyzed by reversed phase HPLC with UV detection. Chromatographic analysis was performed on a TSK ODS-80™ column (150 × 4.6 mm i.d., particle size 5 μm; Tosoh, Tokyo, Japan) in conjunction with a pre-column. The mobile phase consisted of acetonitrile-phosphate buffer (pH 6.8, 20 mM; 60:40, v/v) at a flow rate of 1 ml min^{-1} at ambient temperature. UV detection was set at 295 nm. The peak areas of FK480 and internal standard were estimated automatically by an integrator, and the peak area ratio was calculated. Calibration curves were prepared on the basis of peak area ratios (y) versus concentration of FK480 in plasma (x). A weighted ($1/y$) least squares regression analysis of the data was used to calculate concentrations of FK480 in the validation samples from their measured peak area ratios.

2.3. Preparation of conjugates

Three conjugates of FK480 were prepared: BSA conjugate for immunization, poly-L-lysine conjugate for screening and POD conjugate for competitive EIA (Fig. 1). First, FK480 analog (FR141838) which had been incorporated $-(CH_2)_3COOH$ at the position of the nitrogen atom of the indole moiety was synthesized as follows: 2-Carboxy indole was protected with diphenyldiazomethane in tetrahydrofuran. To the resultant ester was added sodium hydride and t-butyl 4-bromobutyrate in dimethylformamide for the alkylation. Subsequently the alkyl indole ester was deprotected by hydrogenolysis with 10% paradium-carbon in ethanol, and combined with another part of FK480 by the amidation. Finally FR141838 was produced by the

Table 2
Intra-day precision and accuracy measurements of EIA method for FK480

Antibody	Plasma	Concentration spiked (ng ml^{-1})	Concentration found (ng ml^{-1})	n	Coefficient of variation (%)	Bias (%)
PAb	Human	0.1	0.100 ± 0.011[a]	4	11.0	0
		0.5	0.509 ± 0.020	5	3.9	1.8
		2.5	2.40 ± 0.03	5	1.2	−4.0
MAb (2-57-3)	Human	0.2	0.210 ± 0.032	5	15.1	5.0
		1	1.03 ± 0.02	5	1.9	3.0
		10	9.89 ± 0.16	5	1.6	−1.1
	Dog	0.1	0.115 ± 0.013	5	11.3	15.0
		1	0.971 ± 0.029	5	3.0	−2.9
		5	5.13 ± 0.17	5	3.3	2.6

[a]Mean ± S.D.

deprotection of the resultant ester with anhydrous 4 M HCl in ethyl acetate. FR141838 (40 mg, 76 μmol) was reacted with N-hydroxy succinimide (17.4 mg, 151 μmol) and EDC (30.6 mg, 161 μmol) in 5 ml of dichloromethane overnight at ambient temperature to produce activated esterific FK480 (ca 50 mg). The reactions were monitored by thin-layer chromatography. Successively its FK480 (20 mg in 0.4 ml of dioxane, 32 μmol) was conjugated to BSA (100 mg, 1.5 μmol) in 4 ml of dioxane/50 mM phosphate buffer (pH 7.2; 1:3, v/v) at 4°C for 2 days. The poly-L-lysine conjugate was prepared by reacting the activated esterific FK480 (2 mg in 40 μl of dioxane, 3.2 μmol) with poly-L-lysine hydrobromide (10 mg, ca 0.2 μmol) in 0.36 ml of dioxane/5% $NaHCO_3$ (1:1, v/v) at 4°C for 2 days. The POD conjugate was prepared by similar reaction of the activated esterific FK480 (0.25 mg in 5 μl of dioxane, 0.4 μmol) and POD (5 mg, 0.1 μmol) in 0.18 ml of dioxane/5% $NaHCO_3$ (1:1, v/v) at 4°C for 2 days. The POD conjugate solution was then mixed with 0.6 ml of dioxane/H_2O (1:1, v/v) at 4°C for 3 h and 1.28 ml of 0.1% BSA in phosphate buffer (pH 7.2, 50 mM) was added. Each conjugation was purified by dialysis three times against phosphate buffer (pH 7.2, 50 mM). The BSA conjugate solution and poly-L-lysine conjugate solution were made to 10 ml with the same phosphate buffer, and then subsampled into tubes and stored at −20°C. To the POD conjugate solution was added BSA (50 mg) and was made to 5 ml with the dialysis buffer, and then subsampled into tubes to store at −20°C.

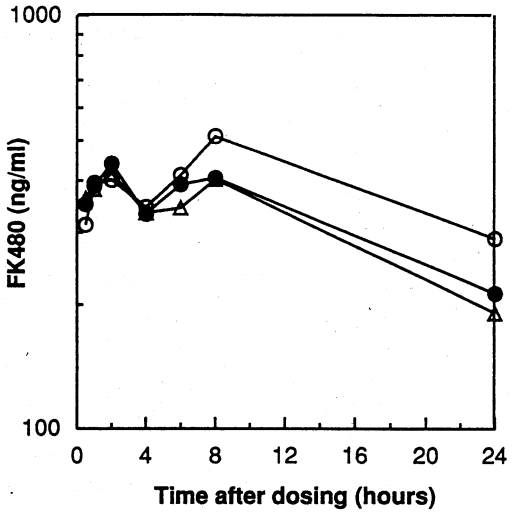


Fig. 2. Comparison of FK480 levels in dog plasma as measured by HPLC (●), PAb EIA (○) and MAb EIA (△) after oral dosing with FK480 at 1 mg kg^{-1}.

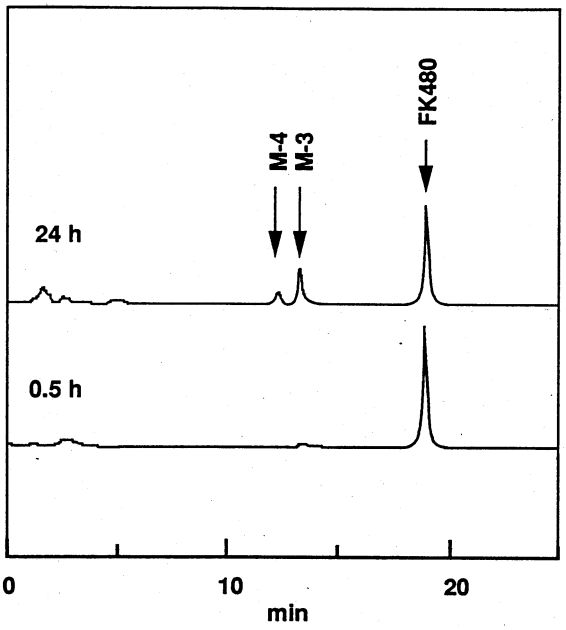


Fig. 3. HPLC chromatogram of dog plasma after oral dosing with FK480 at 1 mg kg^{-1}.

2.4. Production of antisera

Three New Zealand White female rabbits were given subcutaneous injections of approximately 1.7 mg of BSA-FK480 emulsified in Freund's complete adjuvant. Booster injections, emulsified in Freund's incomplete adjuvant and administered intradermally at multiple sites in the back, were then given four times at about biweekly intervals, using the same amount as the dose of the first immunization. Blood samples for testing were taken from the ear vein on day 7 after the third or fourth immunization, and then the rabbits were bled to death from the ear and cervical arteries on day 10 after the final (fifth) immunization to obtain maximum blood. Serum was separated by centrifugation and stored at −20°C until used.

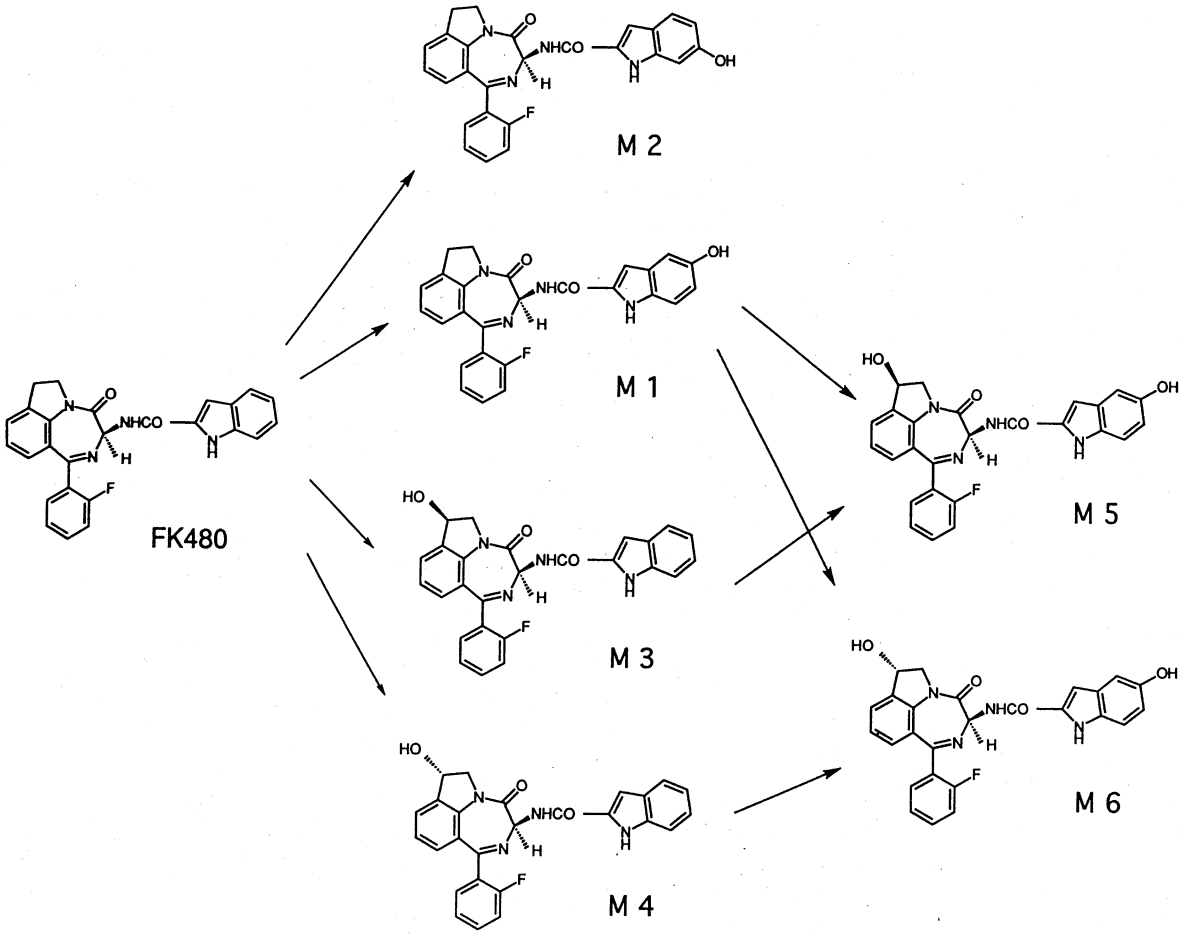


Fig. 4. Postulated metabolic pathway of FK480.

2.5. Production of monoclonal antibodies

2.5.1. Immunization procedure

Five BALB/c mice were immunized with 50 μg of the BSA-FK480 conjugate. The first injection was given intraperitoneally in Freund's complete adjuvant, followed by booster injections emulsified in Freund's incomplete adjuvant (1:1, v/v) on days 10 and 21. Blood samples were collected 8 days after the third injection for titer check. The final injection was given intraperitoneally in saline 4 days before the fusion.

2.5.2. Production of hybridomas

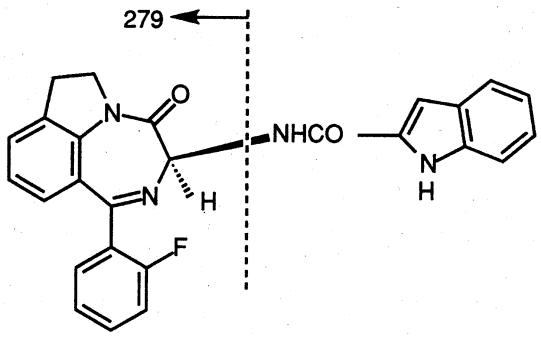
P3-X63-Ag8-653 murine myeloma cells (Dainippon Pharmaceutical Company, Osaka, Japan; ATCC No. TIB-9) were fused with immune spleen cells by conventional hybridization technique as previously described [16]. Briefly, the ratio of spleen cells to myeloma cells was 5:1. They were fused in the presence of 1 ml of 45% PEG 4000 solution. At the end of the fusion procedure, the cells were distributed (1 ml well^{-1}) at a density of 1×10^6 cells well^{-1} into 24-microculture plates. Hybridomas were selected in hypoxanthine-aminopterin-thymidine RPMI medium containing 5% P815 mastcytoma cell (ATCC No. TIB-64) culture supernatants. The supernatants of the growing cultures were screened by ELISA. Antibody-producing hybridomas were immediately cloned by the limiting dilution method.

2.5.3. Immune serum testing and MAb screening procedure

Anti-FK480 antibodies in the mouse serum and in the culture supernatant were detected by their binding to poly-L-lysine-FK480 conjugate. Maxisorp II microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl well^{-1} of poly-L-lysine-FK480 conjugate at 10 μg ml^{-1} in PBS for 2 h at room temperature. The plates were then washed three times with 0.05% (v/v) tween-20 in PBS (PBS-T), and after blocking with 200 μl well^{-1} of 5% skim milk in PBS for 1 h at room temperature, were washed three times with PBS-T. Fifty microliters of the hybridoma supernatants or the mouse serum for testing for anti-FK480 antibody was serially diluted with 1% skim milk in PBS, and added to each well. Then the plates were incubated at room temperature for 2 h. The microtiter plates were washed four times with PBS-T; then 100 μl well^{-1} of peroxidase labeled goat anti-mouse IgG conjugate, at an optimal dilution (10^4-fold) with 1% skim milk in PBS, was added. After incubation for 1 h at room temperature, the plates were washed five times with PBS-T, then 200 μl well^{-1} of 2 mg ml^{-1} o-phenylene diamine hydrochloride, dissolved in sodium citrate—citric acid buffer (pH 5.4, 0.1 M) was added, and the plates were incubated for 10 min in the dark. The reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 and the resulting absorbance was measured at 490 nm with an automated microtiter plate reader.

Table 3
Mass spectral data for major metabolites of FK480

Metabolite	ESI-MS	MS/MS
	$[M+H]^+$	Major fragment ion
FK480	439	279
M1	455	279
M2	455	279
M3	455	295
M4	455	295
M5	471	295
M6	471	295



2.5.4. Preparation of MAbs

Ascites were produced after intraperitoneal injection of cloned selected hybridoma cells into pristane-treated BALB/c mice. MAbs were then purified from the ascites by affinity chromatography on Protein A-Sepharose using MAPS-II kit (Bio-rad, Richmond, CA).

2.5.5. Characterization and selection of specific MAbs

The subclass of the acquired MAbs was determined by using Monoclonal Mouse Immunoglobulin Isotyping Kit (PharMingen, CA). To investigate the antibody specificity against the metabolites of FK480, the EIA using POD-FK480 conjugate was performed as follows: Maxisorp II microtiter plates were coated with 100 μl well^{-1} of anti-mouse IgG at 2.5 μg ml^{-1} in PBS for 2 h at room temperature. The plates were then washed three times with PBS-T. After blocking with 200 μl well^{-1} of 1% BSA in PBS for, the plates were washed three times with PBS-T. A 100-μl of the MAb solution, serially diluted with 1% BSA in PBS (5–640 ng ml^{-1}), was added to each well, and the plates were incubated at room temperature for 2 h. The plates were washed again in the same way as the above procedure, and 50 μl of FK480 standard solution (0.01–10 ng ml^{-1} of 1% BSA in PBS) or isolated metabolites (M3 or M4; 1–1000 ng ml^{-1}) was added to each well, followed by addition of 50 μl of POD-FK480 conjugate at an optimal dilution (2×10^4-fold) with 1% BSA in PBS. After overnight incubation at 4°C, the plates were washed five times with PBS-T. Then the coloring reaction and measurement of absorbance were performed in

the same way as the above screening procedure. The above EIA method was also used to compare the response curve of PAb with that of MAb. In case of PAb the plates were coated with 100 μl well^{-1} of rabbit anti-FK480 serum (diluted 10^4-fold with PBS) for 2 h at room temperature. Skim milk was used for blocking and diluting instead of BSA as in the next section.

2.6. EIA based on polyclonal antibodies

FK480 in human plasma sample was extracted with organic solvent and measured by competitive EIA with POD-FK480 conjugate using rabbit anti-FK480 serum. Maxisorp II microtiter plates were coated with 100 μl well^{-1} of rabbit anti-FK480 serum (diluted 10^4-fold with PBS) for 2 h at room temperature. The plates were then washed four times with PBS-T. After blocking with 200 μl well^{-1} of 5% skim milk in PBS for 1 h at room temperature, the microtiter plates were washed as above. Fifty microliters of 1% skim milk in PBS was immediately added to each well to avoid drying. Subsequently, the sample solutions (100 μl) were added to appropriate wells (duplicate) and the microtiter plates were incubated overnight (ca 16 h) at room temperature. After incubation, the coloring reaction and measurement of absorbance were performed as described above. Data analysis was performed by computer software, SOFT max-J (Wako Chemicals, Osaka, Japan). The dose-response curve was described by the four-parameters equation: $Y = (A - D)/(1 + (X/C)^B) + D$ where X and Y were the FK480 concentrations and its absorbance, respectively, and A, B, C and D were constants.

The sample solution was made as follows: to human plasma (0.2 ml) distilled water (0.8 ml) and 0.1 M NaOH (0.5 ml) were added and the mixture was extracted with 5.5 ml of 2% isoamyl alcohol in n-hexane under basic condition. The mixture was reciprocally shaken for 10 min and centrifuged at ca 2000 g. The upper organic layer (4.5 ml) was transferred to clean glass tubes, and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 200 μl of 1% skim milk in PBS and was mixed with 50 μl of POD-FK480 conjugate (diluted 5×10^3-fold with 1% skim milk in PBS).

2.7. EIA based on monoclonal antibody

This method consisted of extracting FK480 from human plasma sample with 2% isoamyl alcohol in n-hexane under basic condition. The concentrations of FK480 were measured by competitive EIA with POD-FK480 conjugate using monoclonal antibody. Maxisorp II microtiter plates were coated with 100 μl well^{-1} of anti-mouse IgG at 2.5 μg ml^{-1} in PBS for 2.5 h at room temperature. The plates were then washed four times with PBS-T. After blocking with 200 μl well^{-1} of 1% BSA in PBS for 1 h at room temperature, the plates were washed four times with PBS-T. A 100-μl of the anti-FK480 MAb (No. 2-57-3) at 2.5 ng ml^{-1} (diluted with 1% BSA in PBS) was added to each well, and the plates were incubated at room temperature for 2 h. The plates were then washed again as in the above procedure, and the sample solution (100 μl) and 50 μl of POD-FK480 conjugate diluted 2×10^4-fold with phosphate buffer (pH 7.5, 20 mM) containing of 1% BSA and 0.4 M NaCl were added. After incubation overnight (ca 20 h) at 4°C, the plates were washed five times with PBS-T. The POD catalyzed color reaction and data analysis were performed as described for the EIA based on polyclonal antibody procedure.

The sample solution was made as follows: to plasma (0.1 and 0.2 ml for dog and human, respectively), were added distilled water (0.9 and 0.8 ml for dog and human, respectively) and 0.1 M NaOH (0.5 ml), and the mixture was extracted with 5.5 ml of 2% isoamyl alcohol in n-hexane under basic condition. The mixture was reciprocally shaken for 15 min and centrifuged at ca 2000 g. The upper organic layer (4.5 ml) was transferred to clean glass tubes, and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 250 μl of 1% BSA in PBS for dog plasma assay, or in phosphate buffer (pH 7.5, 20 mM) containing 1% BSA and 0.4 M NaCl for human plasma assay.

2.8. Isolation and structure elucidation of metabolites of FK480

^{14}C-FK480 dissolved in PEG 400 was adminis-

tered two times at 32 mg/1.5 MBq kg^{-1} to six male Sprague-Dawley rats. Feces were collected 48 h after the second dosing and homogenized with water. The metabolites (M1–M6) were extracted with acetonitrile followed by diethyl ether from 20% homogenate (500 ml) at pH 10 and purified by prep-scale silica gel column chromatography (Merck, Kiesel gel 60; chloroform-acetone-diethyl amine (80:20:2, v/v/v)) and reversed phase HPLC (Merck, Purospher RP-18 column; 250 × 20 mm i.d., particle size 5 μm). The chemical structures of the metabolites were determined by UV, mass and NMR spectrum data. For investigation of cross-reactivity, metabolites M3 and M4 were also produced by in vitro metabolism. FK480 (150 mg) was incubated with rat hepatic microsomes at 37°C. The incubation mixture contained 100 μM sodium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA and NADPH generation system. The metabolites were extracted with 2% isoamyl alcohol in n-hexane under basic condition and purified by prep-scale silica gel column chromatography (Merck, Kiesel gel 60; dichloromethane-methanol (80:20, v/v)) and reversed phase HPLC (Tosoh, TSK ODS-80™ column; 300 × 7.8 mm i.d., particle size 5 μm). Finally two metabolites (M3 2 mg and M4 0.9 mg) were isolated.

2.9. Cross-reactivity studies

To illustrate the extent of cross-reactivity and parallelism, we plotted the assay response at each concentration expressed as a percentage of the assay response for a sample (B) with a concentration of zero analyte (B_0) over a sufficiently wide concentration range. The concentrations of the isolated metabolites were determined by their ultraviolet absorbance at 290 nm. Cross-reactivity of the metabolites to antibody was further investigated by adding several amounts of metabolites to FK480.

2.10. Dog plasma sample preparation

A capsule of FK480 was administered orally to a beagle dog at 1 mg kg^{-1}. Blood samples were taken at 0, 0.5, 1, 2, 4, 6, 8 and 24 h after dosing into tubes containing sodium heparin as anticoagulant. Plasma was separated and stored at −20°C until analyzed.

2.11. Human plasma sample preparation

Four soft-capsules of FK480 (2 mg) dissolved in cone oil were administered orally to six healthy male volunteers. Blood samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 60 and 72 h after dosing into heparinized vacutainer tubes. Plasma was separated and stored at −20°C until analyzed.

3. Results

3.1. Validation of HPLC

The chromatogram of the plasma from the control dog and drug free volunteers did not show any interfering peak. The calibration curve for FK480 was linear over the range of 3–1000 and 2–50 ng ml^{-1} for dog and human plasma, respectively. The limits of quantification were 3 ng ml^{-1} for dog using 0.5 ml of plasma and 2 ng ml^{-1} for human using 1 ml of plasma. Precision and accuracy measurements are given in Table 1. The coefficient of variation was less than 10% at all concentrations investigated. The absolute recoveries of FK480 by these extraction procedures for dog and human plasma were $103.9 \pm 5.5\%$ ($n = 5$, mean ± S.D.) at 200 ng ml^{-1} and $75.9 \pm 1.6\%$ ($n = 5$) at 20 ng ml^{-1}, respectively.

3.2. Validation of EIA based on polyclonal antibodies

The correlation between the FK480 concentrations and their absorbances was satisfactory, and the measurable range of the calibration curve was between 0.1 and 2.5 ng ml^{-1} for human plasma. The lower limit of quantification (LOQ) by this method was 0.1 ng ml^{-1} when 0.2 ml of plasma was used. Precision and accuracy measurements are given in Table 2. The coefficient of variation was 11.0% at 0.1 ng ml^{-1}, 3.9% at 0.5 ng ml^{-1}

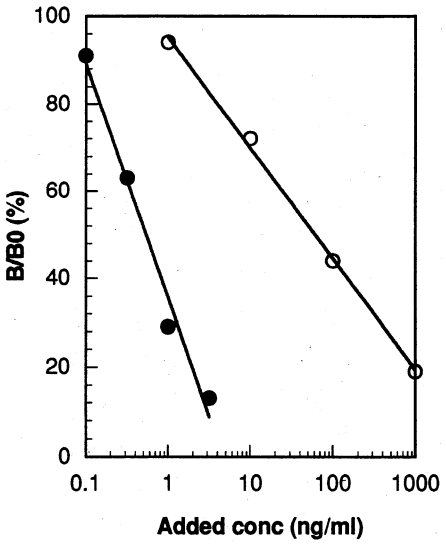


Fig. 5. Response curves of EIA using rabbit anti-serum to FK480 (●) and metabolite M3 (○).

and 1.2% at 2.5 ng ml^{-1}. This method was a hundred times more sensitive than the HPLC method.

3.3. Comparison of two assay methods

After oral administration of FK480 at 1 mg kg^{-1} to dog, the plasma levels were measured by both HPLC and EIA method using polyclonal antibody. The results are shown in Fig. 2. The concentration of FK480 by EIA using PAb were 20–30% higher than that by the HPLC method between 8 and 24 h after dosing. This phenomena suggested that EIA using polyclonal antibody overestimated the actual value owing to the cross-reactivity of the metabolites. We then investigated the HPLC chromatograms given by solvent extraction procedure for EIA (Fig. 3). Two metabolite peaks were observed and were obviously immunoreactive by means of HPLC-EIA (data not shown). These two metabolites (M3 and M4) were produced by in vitro reaction with rat hepatic microsome and isolated for structure elucidation. The structures of M3 and M4 were determined by UV, mass and NMR analysis data. Six metabolites including M3 and M4 were isolated from the rat feces. The postulated metabolic pathway of FK480 is shown in Fig. 4 and mass spectral data are summarized in Table 3. The chromatographic data of M1 and M2 were identified with those of synthetic standards FR189433 and FR189435, respectively.

3.4. Cross-reactivity of the metabolites and specificity of MAb

Three sensitive MAbs (No. 1-4-1, No. 2-47-8 and No. 2-57-3) were selected first by means of EIA using poly-L-lysine-FK480 conjugate. Isotyping techniques revealed that these antibodies were IgG_1 (L_k), IgG_{2a} (L_k) and IgG_1 (L_k) subclass, respectively, though No. 1-4-1 was shown to be weak in affinity to POD-FK480 conjugate (data not shown). Figs. 5 and 6 show the result of cross-reactivity assay using rabbit anti-FK480 serum and anti-FK480 MAbs (No. 2-47-8 and No. 2-57-3), respectively for isolated metabolites M3 and M4. The dose-response curve of FK480 was parallel with those of both M3 and M4 when MAb No. 2-57-3 was used and scarcely showed cross-reactivity ($< 1\%$) to either M3 or M4.

3.5. Validation of EIA based on monoclonal antibodies

The correlation between the FK480 concentrations and their absorbances was satisfactory, and the measurable range of the calibration curve was between 0.1 and 5 ng ml^{-1} for dog plasma and 0.2–10 ng ml^{-1} for human plasma. LOQs were 0.1 and 0.2 ng ml^{-1} using 0.1 and 0.2 ml of dog and human plasma, respectively. Precision and accuracy measurements are given in Tables 2 and 4. Intra-day precision expressed as the coefficient of variation for human plasma was 15.1% at 0.2 ng ml^{-1}, 1.9% at 1 ng ml^{-1} and 1.6% at 10 ng ml^{-1}. Inter-day precision over 3 days for human plasma was 11.9% at 0.2 ng ml^{-1}, 6.0% at 1 ng ml^{-1} and 5.2% at 10 ng ml^{-1}.

3.6. Comparison of EIA using MAb to HPLC method

The concentrations of FK480 in dog plasma by the EIA using MAb (No. 2-57-3) were almost

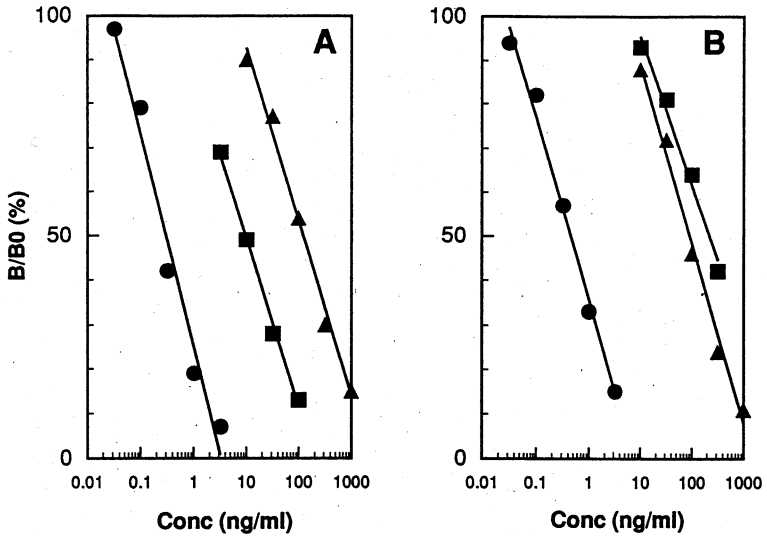


Fig. 6. Cross-reactivity of M3 (▲) and M4 (■) to FK480 (●) using MAb; (A) No. 2-47-8 and (B) No. 2-57-3.

equal to those of the HPLC method (Fig. 2). After oral administration of 2 mg of FK480 to healthy male volunteers, plasma samples were analyzed by EIA using MAb, and by HPLC method. As shown in Fig. 7, the correlation coefficient between EIA and HPLC was good ($r^2 = 0.978$).

4. Discussion

EIA is frequently used for determination of endogenous substances and drugs when a sensitive method is required. However the specificity of the method, especially regarding cross-reactivity of metabolites, is a very important issue when EIA is used to determine the unchanged drug levels for pharmacokinetic study. Part of the dosed drug converts to many structure-liked metabolites by oxidation or phase I metabolism, and the cross-reactive metabolites in the biological fluid cause overestimation of unchanged drug concentration.

A postulated phase I metabolic pathway of FK480 is shown in Fig. 4. Metabolites M3 and M4 appeared on the HPLC chromatograms of dog plasma. We suppose that the cross-reactivities of those two metabolites are the reason why the unchanged drug level by the EIA method was about 20% higher than that by HPLC method. Therefore it was necessary to investigate the cross-reactivity levels and the effect of metabolites M3 and M4 on the EIA method by using isolated metabolites.

Cross-reactivity is frequently expressed as percent rates of the apparent cross-reactant concentration divided by the concentration of the antigen at 50% assay response [17]. But if the response curves are not parallel, the above method is not appropriate for expressing the cross-reactivity [18]. In that case the cross-reactiv-

Table 4
Inter-day precision and accuracy measurements of EIA method using MAb (2-57-3) for FK480 in human plasma ($n = 15$)

Concentration spiked (ng ml^{-1})	Concentration found (ng ml^{-1})	Coefficient of variation (%)	Bias (%)
0.2	0.196 ± 0.023[a]	11.9	−2.0
1	0.970 ± 0.059	6.0	−3.0
10	10.43 ± 0.54	5.2	4.3

[a]Mean ± S.D.

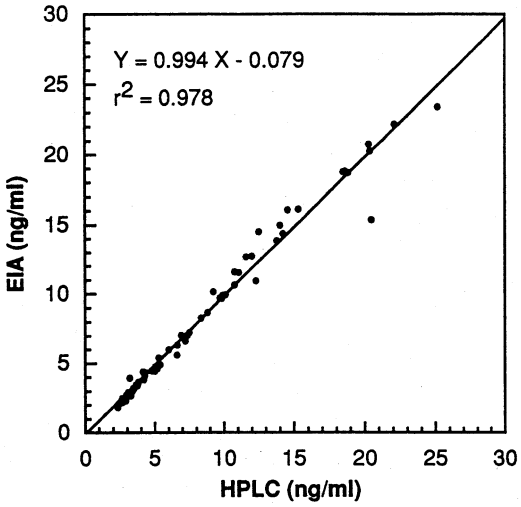


Fig. 7. Correlation of plasma concentrations of FK480 as measured by EIA using MAb and by HPLC in samples from healthy male volunteers ($n = 63$).

ity is measured in the mixture of metabolites and analyte [19]. In this study we evaluated our antibody specificity and effect on the determination values by comparing the responses of FK480 as antigen with and without metabolites as cross-reactants. As it would have been very time-consuming to synthesize metabolites M3 and M4, we used isolated M3 and M4 from in vitro metabolism and calculated the concentration by its absorbance, assuming that to be the same as the UV spectrum of FK480.

The response curves of FK480 and M3 using rabbit anti-FK480 serum were not parallel as shown in Fig. 5. Under coexistence of FK480, the cross-reactivity of M3 was greater than that without FK480. This phenomenon, i.e. synergic effect, is summarized in Table 5. When the concentration of M3 was 1.25 ng ml^{-1}, which was a quarter of the amount of FK480, the apparent concentration of calculating by the response curve of FK480 was 0.127 ng ml^{-1}, so the cross-reactivity was only about 10%. But when FK480 and M3 were combined (the concentration of FK480 was 5 ng ml^{-1} and that of M3 was 1.25 ng ml^{-1}), the determination value was 5.76 ng ml^{-1}. Consequently, since the increasing bias was 0.80 ng ml^{-1}, which was calculated by subtracting 4.96 from 5.76 ng ml^{-1}, the cross-reactivity was 64%. This result showed that the cross-reactivity increased about six-fold owing to the coexistence of FK480. However in the case of MAb the response curves of FK480 and M3 were parallel as shown in Fig. 6. The cross-reactivity of M3 was less than 1%, and a synergic effect was not observed. The mechanism of the synergic effect between antigen and its metabolites as in Table 5 is still unknown.

On the other hand, metabolites M1 and M2 appeared later than M3 and M4 in the plasma after dosing. The cross-reactivity of M1 or M2 to the above monoclonal antibody was very high because the response curve using synthesized M1 or M2 was almost the same as the standard

Table 5
Effect of cross-reactant M3 on determination value of FK480 (5 ng ml^{-1}) by EIA ($n = 6$)

Antibody	Added concentration of M3 (ng ml^{-1})	Found concentration of M3 without FK480 (mean, ng ml^{-1})	Cross-reactivity (%)	Found concentration of M3 with FK480 (mean, ng ml^{-1})	Cross-reactivity (%)
PAb	0			4.96	(100)
	1.25	0.127	10.2	5.76	64.0
	2.5	0.237	9.5	5.81	34.0
	5	0.335	6.7	5.65	13.8
	10	0.482	4.8	6.24	12.8
MAb	0			5.18	(100)
(2-57-3)	1.25	n.d.	—	5.06	—
	2.5	n.d.	—	5.16	—
	5	0.065	1.3	5.27	1.8
	10	0.129	1.3	5.34	1.6

response curve, and suggests that this monoclonal antibody is not able to recognize the indole ring of FK480. As neither M1 or M2 were extracted by the used sample treatment, i.e. extraction of 2% isoamyl alcohol/n-hexane under basic condition, it was clear that these two metabolites did not affect the determination values of EIA. The other metabolites M5 and M6 did not extract since they were more polar than either M1 or M2.

5. Conclusion

In conclusion, we established a sensitive and specific EIA of FK480 using monoclonal antibody in combination with the appropriate sample pretreatment to study the pharmacokinetics of the drug. We believe that this method will be useful in developing FK480 in the clinical stage.

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